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Title: Neutrophil Elastase and Endogenous Inhibitors in Behçet's Disease Saliva

Short Title: Neutrophil Elastase Inhibitors in Behçet's Disease Saliva

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Abbreviations used in this article:

BD, Behçet's disease; BDa, BD patients with active oral ulcers; BDq, BD quiet patients without oral ulcers; RASa, recurrent aphthous stomatitis patients with active oral ulcers; RASq, recurrent aphthous stomatitis quiet patients without oral ulcers; NE, neutrophil elastase; SLPI, secretory leukocyte protease inhibitor; α 1AT, alpha1-antitrypsin; HC, healthy control; OBS, oral buccal swabs (U, ulcerated site; NU, non-ulcerated site); qPCR, quantitative PCR; ECM, extracellular matrix; CatG, Cathepsin G; AZA, azathioprine; COLC, colchicine. WBC, white blood cell.

Summary

Behçet's disease (BD) is a vasculitis of unknown aetiology typified by chronic recurrent oral ulcers and systemic inflammatory manifestations. Neutrophils, and specifically their protease neutrophil elastase (NE), have been implicated in its pathology. Although NE is an effective antimicrobial, excessive NE can damage host tissue. Recurrent oral ulceration is a primary BD symptom, therefore we hypothesized that excessive neutrophil infiltration evidenced by increased NE and a reduction in specific endogenous inhibitors, secretory leukocyte protease inhibitor (SLPI) and alpha1-antitrypsin (α 1AT), contributes to BD mucosal instability. NE, SLPI, and α 1AT were quantified in saliva from BD patients with active oral ulcers (BDa) and quiet without ulcers (BDq), recurrent aphthous stomatitis (RASa; RASq), and healthy controls (HC). Although BDq saliva had marginally higher median NE levels (1112 ng/ml) compared to both RASq (1043

ng/ml) and HC (999 ng/ml), SLPI was significantly reduced in BDq ($p < 0.01$). Despite decreased SLPI protein, mRNA expression was significantly increased in BDq buccal epithelial swabs compared to RASq and HC ($p < 0.05$, $p < 0.001$). NE remained enzymatically active although $\alpha 1$ AT levels were at least eight times higher than SLPI in all groups suggesting $\alpha 1$ AT does not have a primary role in counteracting NE in saliva. Furthermore, NE levels in BDa patients medicated with both azathioprine (AZA) and colchicine (COLC) were significantly lower than those on COLC ($p = 0.0008$) or neither ($p = 0.02$) indicating combining AZA+COLC may help regulate excessive NE during ulceration. This study showed that enzymatically active NE coupled with reduced SLPI in BD saliva may contribute to recurrent oral ulcerations.

INTRODUCTION

Behçet's disease (BD) is a chronic, multi-systemic disorder with evidence of both auto-inflammatory and autoimmune characteristics typified by recurrent oral and genital mucocutaneous lesions (1, 2). Individuals may also experience arthritis, eye lesions, gastrointestinal issues, vascular inflammation, and/or neurological disorders. Although the mechanism of pathogenesis is unknown, there is some evidence of autoimmunity by way of anti-endothelial cell antibodies as well as immune dysfunction with a genetic predisposition to the human leukocyte antigen complex, HLA-B51 (3-5). Oral ulceration occurs in all BD patients typically preceding systemic symptoms suggesting these ulcers are a key event in this disease (6). Morphologically, BD ulcers are similar to recurrent aphthous stomatitis (RAS) with a few differentiating characteristics (7). In BD, a large swollen peri-ulcer with erythema is visible. Majors ulcers tend to be destructive with scars distorting the oral mucosa (8). In RAS, peri-ulcer erythema is reduced and quickly diminishes as the ulcer heals from the base. During quiet episodes, BD patients frequently still have erythematous mucosal areas. Histologically, BD and RAS ulcers share similar features (9). Biopsies show infiltration of neutrophils, macrophages, T cells, and proinflammatory cytokines (1, 10). However, interleukin (IL)-4 has only been detected in BD ulcers (11). The dichotomy of IL-4 is that it has been shown to down-regulate neutrophil migration (12), but can also delay neutrophil apoptosis and induce IL-8, a potent neutrophil chemoattractant (13). There is a lack of research regarding the possible mechanisms that are involved in the induction of BD oral manifestations despite the fact that the initial onset of oral ulceration precedes all other symptoms.

Neutrophil chemotaxis and infiltration is evident in BD oral, ocular and cutaneous lesions and has been implicated in the disease's progression and pathology (14-16). Gene HLA-B51, having the strongest association with BD (5), was also found to be associated with neutrophil hyperactivity (17). Treatment is aimed at preventing systemic reactivation. Immunomodulatory medication, such as colchicine, is used in patients with recurrent mucocutaneous BD to reduce neutrophil chemotactic activity (18).

Neutrophil elastase (NE) is a serine protease produced mainly by promyelocytes (19). NE is contained in azurophilic granules alongside Cathepsin G (CatG) and Proteinase-3 in a ready-to-use state. The controlled mechanism of NE delivery consists of either the release of granules into a phagolysosome where microbes are degraded, or exocytosis of the azurophil into extracellular spaces (20). Uncontrolled release of NE can also be due to necrosis and/or NETosis (21, 22). Neutrophil degranulation can be triggered by various inflammatory factors or direct contact with the extracellular matrix (ECM) (23) and increased neutrophil recruitment can extend the duration of inflammation (24). High extracellular NE levels in chronically inflamed tissue of individuals with chronic obstructive pulmonary disease (COPD) (25) and inflammatory bowel disease has been implicated as a cause of tissue damage (24). Being kinetically faster than CatG and Proteinase-3 (26), NE is capable of quickly degrading ECM components such as elastin, collagen, and fibronectin (24). Therefore, NE regulation is vital.

Secretory leukocyte protease inhibitor (SLPI) has been reported as one of the primary regulatory inhibitors of NE in COPD patients' sputum (27). SLPI is a stable innate inhibitory protein which has broad spectrum anti-microbial activity and protects mucosal epithelial surfaces from proteolytic enzymes such as NE, CatG, and trypsin (28). SLPI is produced in many cell types including saliva gland acinar cells and mucosal epithelial cells (29) and has been detected in various secretions including bronchial fluid (30) and saliva (31).

Alpha1-antitrypsin (α 1AT), a systemically circulating serine protease produced mainly in the liver, is a well-known inhibitor of NE in the lung. It has been measured in saliva (32) and gingival crevicular fluid in periodontal lesions (33). Notably, both SLPI and α 1AT have also been identified in neutrophil cytoplasm separated from the enclosed NE granule suggesting neutrophils have evolved to protect themselves from their own potentially damaging enzymes (28, 34).

Neutrophil infiltration is evident in BD oral ulcers, and uniquely, IL-4 which could delay neutrophil apoptosis. Degranulation is triggered by various inflammatory factors such as TNF- α , IL-8, C5a and LPS (35) releasing excessive NE into the oral environment which can quickly degrade epithelial tissue (36). The aim of this study was to clarify the role of NE and its endogenous inhibitors in the oral cavity which may help elucidate the mechanisms by which mucosal ulceration occurs in BD. It was hypothesized that BD patients have insufficient levels of salivary SLPI and α 1AT enabling excessive extracellular NE to damage the oral epithelial mucosa leading to cellular instability and recurrent ulcerations. The information deduced from this research may help improve targeted drug therapy to decrease recurrent episodes, increase the healing rate, and ultimately prevent ulceration.

MATERIALS AND METHODS

Patients and saliva sample collection

This study was approved by the local research ethics service of the City and East London (P/03/122). All subjects gave written consent and all samples were collected between 01/2009–12/2012. STROBE cohort guidelines were followed. BD patients fulfilled the International Study Group Criteria (37). Although BD is a multisystem disorder, with a broad range of clinical manifestations, we focused on the oral environment and therefore in the manuscript refer to an active oral episode (BDa) as the presence of at least one oral ulcer at the time of sampling regardless of size, duration, or other clinical symptoms. BD and RAS ulcers can resolve and reoccur intermittently. If no oral ulcer was present at the time of sample collection, regardless of other systemic symptoms, the individual was deemed orally quiet, or BDq. A separate questionnaire for BD patients' systemic symptoms was recorded on the day of their clinic. Evidence of disease activity at different sites such as their skin, genitals, or joints was recorded separately as quiet or active (**Table S1**).

Saliva was collected from BD patients with active oral ulcers (BDa, n=93) and those without oral ulcers deemed BD quiet (BDq, n=103) at monthly-held Behçet's daytime clinics at the Royal London Hospital. RAS subjects with active oral ulcers (RASa, n=19), RAS without ulcers (RASq, n=16) and healthy controls (HC n=82) were recruited using local flyers. Participants rinsed with 5

ml of water for 10 seconds and expelled. Passive drooling of 1-5 ml of unstimulated saliva was collected into a sterile container, transferred to the laboratory on ice, and centrifuged at 3500g for 15 min at 4°C. Supernatants were aliquoted and stored at -80°C until further analysis.

Salivary levels of SLPI, NE and α 1AT

Enzyme linked immunosorbent assays (ELISA) were used to measure human SLPI (Quantikine®, R&D Systems, UK), NE (HK319, HyCult Biotech, The Netherlands) and α 1AT (Genway Biotech, Inc, USA) in saliva following the manufacturers' protocols. **Table 1** summarizes the number of BD, RAS and HC saliva samples tested per assay. Saliva was diluted 1:200 in PBS and run in duplicate alongside standards. Initial validation experiments were carried out for each ELISA. This involved adding recombinant or purified target protein to ELISA wells alone or in the presence of BD saliva in order to assess the accuracy of the assays. Each ELISA successfully recovered the spiked amount at a 1:200 saliva dilution (acceptable recovery being between 80 – 120%).

Elastase activity in saliva samples

Total protease activity as well as NE-specific enzymatic activity in saliva was measured using the EnzChek® Elastase Assay Kit (Molecular Probes/Life Technologies, UK) as per the manufacturer's instructions. The following patients' saliva samples were tested: BDa n=7, BDq n=6, RASa n=4, RASq n=5, and HC n=8. First, BDa and RASa saliva samples were diluted 1:20 using the 1X reaction buffer and those from BDq, RASq, and HC's were diluted to 1:10. An aliquot of 100 μ l of these diluted samples was added in duplicate to a 96-well plate containing 50 μ l of 800 μ M NE inhibitor (N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) or 50 μ l of 1X reaction buffer for the non-inhibited reaction. Next, 50 μ l of 100 μ g/ml of the DQ™ Elastin conjugated with BODIPY® FL dye was added to each well to bring the final volume to 200 μ l. Porcine elastase was provided as the protease activity positive control. Its reaction consisted of the same volume of DQ™ Elastin with and without inhibitor. A negative control with only reaction buffer and DQ™ Elastin was also included. With an excitation wavelength of 480 nm, emitted fluorescence at a wavelength of 520 nm was detected on an OPTIMA FLUOStar spectrophotometer (BMG Labtech, Germany) and recorded every 5 min for a total of 95 min at 37°C. An increase in fluorescence during 95 minutes indicated high elastase activity in the saliva. After adjusting for sample dilutions, the difference between neutrophil-specific elastase activity

and total elastase activity (i.e. with and without inhibitor) was calculated in order to determine the NE-specific activity in each saliva sample.

Oral buccal swab sample collection

A swab of oral buccal epithelial cells was collected from all participants in order to determine the extent of the local expression of SLPI mRNA other than from the saliva glands' acini. Therefore, after saliva was collected, oral buccal swabs (OBS) were collected from HC, RAS, and BD patients by brushing the inside of the cheek sweeping up and down 10 times using a nylon cytology brush (Deltalab, Spain). If sloughed from an ulcerated site, the sample was denoted as "U" and if from a non-ulcerated site "NU". Gentle pressure was used for obtaining swabs from ulcerated sites in order to minimize discomfort to patients. The brush was then vigorously washed in 400µl of RLT Buffer (Qiagen, UK). The samples were frozen on dry ice and transferred to -80°C.

Extraction, purification, and reverse transcription of RNA

OBS were defrosted on ice and RNA extracted using the RNeasy Mini Kit (Qiagen, UK) as described by the supplier's protocol followed by DNase enzyme digestion using TURBO™ DNA-free kit (Ambion, UK). RNA quantity and quality were assessed using Nanodrop™ 1000 (Thermo Scientific, UK). A ratio of 1.7-2.0 indicated good quality RNA. Reverse transcription (RT) was performed on ≤ 2 µg per 20 µl reactions using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, UK) as per the manufacturer's instructions. Each 0.2 ml reaction tube was placed into a Veriti Thermocycler (Thermo Scientific, UK) with the following incubation times and temperatures: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min to deactivate the RT enzyme, and cooled to 4°C. The new complimentary (c)DNA was aliquoted and kept at -20°C.

OBS reference gene panel

The Real-Time ready Human Reference Gene 384-well Panel (Roche, Germany) was used to test a panel of 19 potential reference genes in a total of 48 OBS from BD, RAS, and HC subjects. The manufacturer's protocol was followed. For our plate set up, each cDNA sample was first diluted 1:10 in RNase-free water to a final volume of 125 µl. To this, 125 µl of 2X LightCycler® 480 Probes Master reaction mix (Roche, Germany) was added. After mixing, 10 µl was pipetted into

each well across an individual row (columns 1 – 23), i.e. each row consisted of one patient sample. A total of 16 patient RNA samples were applied to one plate. Column 23 and 24 (the RT-negative controls) each had 1 µl of the previously set aside non-transcribed OBS RNA sample, 4 µl of water, and 5 µl of the 2X LightCycler® 480 Probes Master reaction mix. Plates were sealed, centrifuged briefly, and run on the LightCycler® 480 System I instrument (Roche) by uploading the company provided macros program.

SLPI and PPIA qPCR optimization

All primers were designed based on human mRNA database sequences taking into account any multiple transcript variations. SLPI (accession NM003064.2) primers were designed by the Universal Probe Library program (Roche Applied Science, UK): SLPI F-primer:

5'CTGTGGAAGGCTCTGGAAAG and SLPI R-primer 5'GGCACTGGGCAGATTTCTTA.

Primer design for one of the most stable reference genes, peptidylprolyl isomerase A (PPIA, accession NM021130), was selected from RTPrimerDB: (F-primer

5'TCATCTGCACTGCCAAGACTG, R-primer 5'CATGCCTTCTTTCACTTTGCC). Primers were manufactured by Sigma-Aldrich, UK.

During quantitative (q)PCR assay optimization, primers underwent an initial reaction with 2X Sybr Green I Mastermix (Roche, UK) and at least 2 different cDNA OBS samples to ensure amplification of one product. In order to assess this, the melt temperature (T_m) of the amplicon was completed and water (negative) controls were run every time and monitored for any amplification. Single amplicon product sizes, 66 bp for SLPI and 71 bp for PPIA, were confirmed by DNA electrophoresis on a 1% agarose gel. Purified cDNA was subsequently used for standards. Since a pre-mixed 2X Sybr Green I Mastermix was used for all reactions, dNTPs, Taq polymerase, and $MgCl_2$ concentrations were all pre-determined by the manufacturer and therefore special consideration was spent on forward and reverse primer concentration as well as annealing temperature and time in order to optimize the individual assays.

The final qPCR assays for each target consisted of 400nM forward and reverse primers with 2X Sybr Green I Mastermix (Roche, UK) and 5 µl of cDNA in a final 12.5 µl reaction. Both assays were optimized to run at the same temperature cycles which included initial activation of Taq polymerase at 95°C for 5 min followed by 45 cycles of 95°C for 10 sec, 65°C for 10 sec, and 72°C

for 10 sec on a LightCycler® 480 System I instrument (Roche, UK). A total of 86 OBS samples were analyzed for SLPI/PPIA mRNA (HC n=16; BDq n=27, BDa, NU n=20; BDa, U n=6; RASq n=11; RASa, NU n=4; RASa, U n=2).

Statistical analysis

IBM SPSS v23 was used to analyze ELISA and enzyme kinetics data using the non-parametric Mann-Whitney U test. Significance was based on 2-tailed, 95% confidence intervals (CI) where * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$. Spearman's rho with 2-tailed significance was used for correlation analysis. QBasePlus software V2.1 (Biogazelle, Belgium) was used for normalization of qPCR data followed by analysis using an unpaired t-test with Welch's correction on GraphPad v.7.03.

RESULTS

Salivary SLPI, NE and α 1AT levels

BD and RAS patients with ulcers had significantly higher NE levels compared to those without ulcers. **Figure 1A** shows that BDa saliva (1794 ng/ml, median) and RASa saliva (3080.7 ng/ml) were significantly higher than all other groups without ulcers: BDq (1111.7 ng/ml, $p < 0.05$), RASq (1043.4 ng/ml, $p < 0.05$) and HC (998.9 ng/ml, $p < 0.01$). The median NE level in the BDq group was higher than HC and RASq but not significantly so.

However, SLPI was significantly reduced in BDq patient's saliva (198.7 ng/ml, $p < 0.05$) compared to HC and RAS and had the lowest median salivary SLPI levels amongst all groups (**Figure 1B**).

BDa and RASa had similar levels of SLPI at 320.6 and 383 ng/ml, respectively, however only BDa saliva was significantly lower than RASq ($p < 0.01$). SLPI levels were highest in HCs (487.3 ng/ml) and RASq (860.1 ng/ml) patients. Spearman's rho correlation analysis of $n=37$ BDq patients whose saliva was tested for both SLPI and NE showed a diminished inverse relationship

($r = -0.195$, $p=0.25$) compared to HC ($r = -0.419$, $p=0.02$) and BDa ($r = -0.299$, $p=0.09$) (see Suppl. **Figure S1**.)

Salivary α 1AT levels were significantly higher in BDq (8395.3 ng/ml) compared to HC (4995 ng/ml, $p<0.05$) (**Figure 1C**). BDa α 1AT (14,326.1 ng/ml) was nearly double the quantity of BDq and significantly higher than HC ($p<0.001$) but not BDq. RASa and RASq had higher α 1AT levels than HC, but neither was significant.

Although the focus was on investigating NE, SLPI, and α 1AT levels in saliva, BD patients were also clinically assessed for other systemic symptoms (**Table S1**). BDq patients whose saliva was tested for NE, SLPI, and α 1AT also commonly had painful joints (19, 21, 38%) and folliculitis (12, 14, 14%), respectively. The median saliva NE level for BDq patients experiencing joint pain and folliculitis were both 1843.6 ng/ml. There was no significant difference in the BDq salivary NE concentration when these two sites were recorded as clinically active (**Figure S2. A**). The median SLPI saliva concentration for BDq with joint pain was 147.3 ng/ml while those with folliculitis was 367.7 ng/ml. Again, there was no significant difference in the saliva levels when these two most common sites were active (**Figure S2. B**). However, when BDq had active joints ($n=11$), the α 1AT in their saliva was significantly higher ($p=0.02$) (**Figure S2. C**). For BD patients with active oral ulcers the most frequent ailments were folliculitis (34-46)%, followed closely by joint pain (33-44%), and genital ulcers (25-33%) (**Table S1**). The NE, SLPI, and α 1AT saliva levels in BDa patients were not significantly higher when they were experiencing folliculitis or joint pain (**Figure S2. D-F**),

Salivary NE levels and patient medications

While no systemic medications were reported for HC or RAS groups, similar numbers of BDa and BDq patients were receiving immunomodulatory medications at the time of sample collection (**Table S2**). Saliva samples were chosen from this cohort based on active or quiet ulcer status. The medications were analysed as a secondary variable.

Salivary NE levels were measured in both BDa and BDq patients taking colchicine (COLC) or azathioprine (AZA), both, or neither COLC or AZA (**Figure 2**). Although there was no significant difference in NE levels among BDq patients, those who were taking both colchicine and

azathioprine tended to have increased NE (2309 ng/ml). However, the NE levels in BDa patients on *both* COLC and AZA were significantly lower (1131 ng/ml) compared to those taking only colchicine (1816 ng/ml, $p=0.0008$) or those on neither COLC or AZA (2141 ng/ml, $p=0.02$). This group of BDa patients on COLC and AZA had similar NE levels to healthy controls (999 ng/ml, **Figure 2A**) and although this group consisted of a small number of patients ($n=7$), they exhibited a narrow range of NE levels (490–1347 ng/ml).

Enzymatically active NE in saliva

Elastin-specific proteases were measured, and HC saliva had the lowest overall protease activity compared to all other study groups (**Figure 3A**) based on an equal amount of elastin substrate being added to each saliva sample. Within 95min of exposure to the substrate, the total protease and NE enzyme activity was significantly higher in both BDa and BDq patients compared to HC ($p<0.05$). When comparing each group's NE activity relative to their total protease activity, there were no significant differences (**Figure 3B**). This was determined by dividing the median fluorescence of the NE-specific activity by the total protease activity fluorescence for each group and multiplying by 100. The NE in HC saliva comprised ~78% of the total protease activity. Similarly, RAS and BD saliva samples also showed that 79.6% and 82.8% of their total protease activity respectively was due to NE.

Stable reference genes in OBS

Duplicate SLPI Cq values were averaged and normalised (Calibrated Normalized Relative Quantity) using the qBasePlus (Biogazelle software, Belgium) against the Cq values for the PPIA reference gene. Out of 48 OBS, 13 samples were excluded from analysis due to a final Cq value > 40. RASa, U samples were completed but not included in analysis due to the small sample size, only $n=2$. None of the RT-negative samples amplified and were therefore clear of any gDNA contamination. The four most stable reference genes validated for BD, RAS, and HC OBS were peptidylprolyl isomerase A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), and beta-actin (ACTB) (**Figure S3**). PPIA primers performed the most efficiently and due to the limited amount of material for multiple assays, this reference gene was used to normalize the qPCR results for SLPI mRNA expression.

SLPI mRNA expression

SLPI mRNA was significantly increased in BDa, NU ($p \leq 0.01$) and BDa, U ($p \leq 0.05$) when compared to HC (**Figure 4**). Also, SLPI mRNA was significantly upregulated in BDq ($p \leq 0.001$) compared to HC and RASq ($p \leq 0.05$). Unfortunately, the only two RASa, U samples available showed a wide variable range and were therefore excluded from final analysis.

DISCUSSION

Increased circulating neutrophils and their degranulated enzyme, NE, have previously been implicated in the systemic chronic inflammatory response in BD (14, 15). The recurrent oral ulcers that accompany BD may also result from unregulated NE-stimulated inflammation. As observed in a previous study, it was thought that protective salivary SLPI would increase during oral ulceration since it has been shown to aid wound healing (38). We report here for the first time that during quiet oral episodes in which BD mucosa is not ulcerated, individuals with BD have active proteolytic NE in their saliva in quantities similar to HCs and RASq subjects; however, levels of SLPI in BDq are significantly lower. Taken together these results indicate that the presence of active NE and reduced SLPI may be contributing factors to initiation of BD oral ulceration (**Figure 5**). Another endogenous inhibitor, $\alpha 1$ AT, despite its abundance in saliva, does not appear to affect NE activity. The results suggest that there is an association between high levels of proteolytic enzymes and oral mucosal instability leading to oral ulceration. Furthermore, our data shows that BDa patients medicated with both AZA and COLC have reduced salivary NE.

The Inverse Relationship of NE and SLPI

All individuals had quantifiable NE and SLPI in their saliva. Our data shows a ratio of 2:1 NE:SLPI concentration based on collective HC median values using an ELISA with monoclonal

antibody detection of each protein. While NE increased during oral ulceration in RAS and BD patients, BDq patients had only slightly higher NE levels than HC's, but showed a significant reduction of SLPI (the lowest of all groups) giving a concentration ratio of 5:1 NE:SLPI – precisely the same NE:SLPI ratio as BDa levels. This suggests that SLPI is depleted in BDq patients despite being orally asymptomatic. A similar inverse NE/SLPI dynamic has been previously reported in active periodontitis saliva (39) and reduced SLPI has been associated with periodontal disease progression (40).

SLPI has two structural domains allowing the binding of two different targets (29). We therefore postulated that since SLPI derived from whole saliva could simultaneously bind to both NE and a different second target (such as a microbe) this could block SLPI detection by ELISA antibody. To further validate and scrutinize the reduction of SLPI in our BDq cohort, we conducted a spike-recovery ELISA experiment. After adding known concentrations of recombinant (r)SLPI into BDq saliva in which SLPI had previously been measured, the detection recovery of rSLPI was between 89 – 99%. This provided us with evidence that the ELISA detection was accurate despite the possibility of SLPI to be masked while exposed to multiple targets in saliva.

Other reasons for the depletion of SLPI in saliva may involve cysteine proteases such as cathepsins or chymase, a serine protease from mast cells, which can cleave and inactivate SLPI (39). NE has also been shown to directly degrade SLPI which could explain the inverse concentration levels (41). Another reason for the lower SLPI concentration could be due to its direct attachment to oral microbes. There is strong evidence that SLPI has broad spectrum antimicrobial activity (29). In a study by Seoudi, *et al*, the oral bacterial profile of BD and RAS patients with and without ulcers and HCs revealed that the different subject groups had different bacterial species colonization (42). Microbial flora has been previously implicated in BD pathogenesis (43) which may further explain their decreased levels of SLPI.

Increased SLPI mRNA but decreased SLPI protein is characteristic during BDq

Reduced SLPI levels could also indicate that sufficient levels of SLPI were not available in the first place. This would be especially detrimental in an inflammatory condition such as BD in which regulation of pro-inflammatory factors is vital to reduce symptoms. This prompted us to investigate local SLPI mRNA expression. Both saliva gland acini and epithelial cells contribute to salivary SLPI levels. Excising patient gland cells was unrealistic, so we instead investigated SLPI

mRNA expression in the oral epithelium using buccal swabs. The vast majority of studies investigating oral buccal epithelial cell RNA involves oral squamous cell carcinoma and many do not mention validation of their reference genes. To date, this is the first study to report stable RNA reference genes in OBS from HC as well as BD and RAS patients with and without oral ulcers and is explored in depth in the Supplement Part II.

The expectation was that SLPI mRNA would be reduced reflecting the low salivary levels. Surprisingly, the results indicated that SLPI mRNA was significantly upregulated in BDq patients' buccal epithelial cells. A similar paradox was reported in an *in vitro* experiment by Sallenave, *et al*, in which NE was used to stimulate A549 epithelial cells. After 24 hrs, SLPI protein levels were decreased by 94% while SLPI mRNA was upregulated (44). The rate of translation (mRNA to protein), production, and cellular exocytosis of SLPI is unknown for buccal epithelial cells, however *ex vivo* human upper respiratory tract submucosal cells have shown a delay of SLPI exocytosis after NE exposure (45). We speculate that once SLPI is available in the extracellular space in the presence of high levels of NE, it is rapidly utilized.

In BDa patients, the upregulation of SLPI mRNA could be directly stimulated by high levels of salivary NE. However, what is decisively characteristic is that BDq patients with no oral ulceration have the highest SLPI mRNA expression, lowest salivary SLPI, and NE levels on par with HC and RASq. This suggests that BDq patients are likely more sensitive to NE stimulation in comparison to HC or RAS patients. Also, the SLPI mRNA data provides compelling evidence of a differentiation between the oral mucosal environment of BDq and RASq patients.

A small portion of the BDq patients in this study presented with other systemic clinical manifestations such as joint pain, skin lesions, and genital ulcers at the time of their saliva donation demonstrating their underlying systemic inflammatory activity. Although the numbers were small, it was found that only salivary α 1AT levels were significantly increased in BDq patients with active joint pain ($p=0.02$). This could be due to increased α 1AT production by the liver and vascular dissemination. None of the BDa patients showed any significant difference in their NE, SLPI, or α 1AT saliva levels when assessing their most common systemic symptoms. Our data would suggest that NE and SLPI in saliva do not necessarily reflect other site-specific

systemic activity. Therefore, importantly, when assessing SLPI as a biomarker in BDq patients, other systemic symptoms should not greatly influence the oral concentration.

Even though our data did not show significantly higher NE saliva levels during other active systemic symptoms, increased circulating peripheral blood neutrophils are likely to influence tissue and oral infiltration. A study by Deger, *et al*, showed elevated levels of NE in plasma from 22 BD orally quiet patients compared to HC (14). Further studies are required that measure NE, SLPI and α 1AT levels in matched serum and saliva of BD patients to explore if there is a more robust correlation with clinical activity in the mouth, eyes, skin, joints and CNS.

Lakschevitz, *et al*, have reported that oral and peripheral blood neutrophils from HC's behave differently. Based on microarray data, they found that HC salivary neutrophil RNA changed their transcriptome when they migrated from the blood to the oral environment. This alteration was especially evident during increased oral infection or inflammation (46, 47) It has also been suggested that neutrophils are already primed by chemotactic factors such as IL-8 or TNF- α by the time they reach the oral cavity (48). There is, therefore a need to keep in mind that oral neutrophils are exposed to different microbial, antigen and toxin conditions in saliva when compared to blood, which can influence the release of potent anti-microbial proteases such as NE into the saliva and oral cavity (49).

Importantly, during quiet oral episodes, increased NE degranulation in saliva would likely rely on local anti-protease activity such as SLPI to curtail epithelial damage. Our data suggest that SLPI's depletion in BDq saliva may be a key factor in the manifestation of oral ulcers. It may benefit BD patients to use a local, topical NE-inhibitor such as a compound containing rSLPI to help control recurring oral ulceration (50).

Salivary Proteases and NE Activity

NE was confirmed to be enzymatically active in saliva and responsible for the majority of protease activity. It was anticipated that patients with ulcers would have increased NE activity since the ulcerated wound would have initiated neutrophil recruitment to the site. However, BDq and RASq also had higher NE activity compared to HCs. Individuals with BD have been described as having hyperactive circulating neutrophils (16). Our data adds to this observation that proteolytic, active

oral NE is also present. Under-regulated, enzymatically active NE in BD saliva is likely to contribute to recurrent mucosal damage and ulceration characteristic of BD.

α 1AT is Not the Main NE Inhibitor in Saliva

NE and α 1AT showed a direct relationship – if NE increased, α 1AT also increased. In HCs, α 1AT was five times the NE concentration and approximately ten times that of SLPI. SLPI and α 1AT are competitors in that they both inhibit NE. Both also reside inside neutrophils (34) presumably protecting neutrophils from unintentional self-induced proteolytic damage. The majority of α 1AT is produced in liver cells. Approximately 1-2 g/L is transferred to plasma and up to 10% diffuses into biological fluids such as saliva (51). As a serpin, α 1AT is thought to irreversibly bind and inactivate NE (52), yet in our enzyme assay, salivary NE was proteolytically active. This leads us to deduce that despite the abundance of α 1AT in saliva, it is not the main inhibitor of NE in the oral cavity. Also, various proteases can inactivate α 1AT such as collagenase (53), therefore, it is also possible that α 1AT's inhibitory site, which is susceptible to oxidation, is inactivated by reactive oxygen species generated by phagocytic cells and bacteria in saliva (54).

Colchicine-Azathioprine May Lower NE during BD Ulceration

The combination of COLC and AZA medications is associated with decreased NE in BDa saliva. COLC is one of the recommended medications for treating mucocutaneous lesions by reducing the migration of neutrophils (18) and has been shown to modulate oxidative stress in BD neutrophils by regulating Ca^{2+} release (55). In combination with AZA, we sought to determine whether this combination of systemic medication influences oral NE levels. We found that BDa patients taking both medications had significantly lower NE which was surprising during ulceration. Despite the small sample size, the narrow range of their NE measurements indicates low variability. AZA is an immunosuppressive drug which lowers the proliferation of lymphocytes (56). Colchicine accumulates in WBC's (namely neutrophils) disrupting various inflammatory activation, degranulation, and migration processes (57). Coupled together, the drugs' synergistic effects of reducing WBC's and proinflammatory activities, thus decreasing NE degranulation, may help to control inflammation. Further investigation is necessary to validate this data.

Study Limitations

There are some limitations to this study. First, RAS subjects were sought as a non-systemic disease controls from general oral medicine clinics and from local adverts, but recruitment was lower than expected compared to our BD cohort which was recruited from dedicated specialist BD clinics. Individuals with RAS are thought to endure a unique immune dysregulation which, for unknown reasons, manifests solely in the oral cavity (58). Furthermore, Ueta, *et al*, have suggested that individuals with RAS may harbour an immune cell dysfunction based on the evidence that their neutrophils had suppressed superoxide production compared to HCs (59). Overall, it was rationalised that the RAS group, although having small patient numbers, would still add further important insights into oral ulceration mechanisms for which there is very little known.

Another limitation presented itself when directly swabbing mucosal ulcers too gently with cytology swabs as this did not generate enough RNA for all analyses. In future collections, a softer swab that would be both gentle to patient's ulcerated mucosa and compatible with PCR assays would be used. Another consideration for future research would be to collect data on the subject's smoking status, race, duration of treatment, and alterations in treatment regimens. Also, relatively large volumes of saliva were necessary to run NE, SLPI, α 1AT and enzyme activity assays in duplicate. Therefore, it was not possible to run all the analyses on the same set of saliva samples which resulted in unequally sized groups for the different assays. Although BD is a rare disorder, this research study benefited from a substantial number of participants thanks to their attendance and recruitment at the Royal London Hospital's specialized clinic.

In conclusion, this study was able to describe the depletion of SLPI in BD saliva during orally asymptomatic episodes and highlights its potential in balancing the damage caused by enzymatically active, excessive NE. As biomarkers, the collective measurements of NE and SLPI in saliva alongside oral epithelial SLPI mRNA could differentiate between RASq and BDq to assist clinical evaluation. Early intervention could be key to controlling the progression of inflammation associated with BD.. During oral ulceration, the current combined treatment with colchicine and azathioprine seems to lower NE in saliva which could help control inflammation. As a local, topical treatment, recombinant SLPI is commercially available and has been trialed in other inflammatory disorders (50, 60, 61). This study also found that α 1AT, the most well-known NE-inhibitor in the vasculature, did not seem to translate its anti-protease activity in saliva – again highlighting the importance of SLPI as the local NE inhibitor.

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Author contributions were as follows: T.N. and E.H-P. contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. F.F. and L.B. helped with the analysis and interpretation as well as a critical review the manuscript. I.K. assisted with data acquisition and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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All authors confirm there are no financial conflicts of interest.

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Figure Legends

Figure 1. Saliva Protein Levels Amongst Patient Groups. Tukey boxplots show **A.** NE **B.** SLPI, and **C.** α 1AT levels in HC (healthy control), RAS (recurrent aphthous stomatitis), and BD (Behçet's disease) patients. **q**, indicates quiet episode/no ulcers. **a**, refers specifically to at least one active mouth ulcer regardless of other systemic symptoms. Each saliva sample was measured in duplicate. Mann-Whitney U test was used to assess differences between two groups at a time. Significance based on 2-tailed, 95% CI. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. Saliva NE levels of BD patients taking systemic colchicine and/or azathioprine during quiet or active oral ulceration. The Tukey box plots show ELISA NE levels in the following groups: BDq patients with no oral ulceration at the time of sampling and BDa patients who had oral ulcer(s). Patients were identified as taking colchicine (COLC) or azathioprine (AZA), both, or neither AZA or COLC. "COLC only" refers to the exclusion of AZA and vice versa, however, does not exclude other medications the patient may have been taking. Mann-Whitney U test, significance based on 2-tailed, 95% CI. Exact p values are indicated. NS, no significance.

Figure 3. Although the quantity of neutrophil elastase differs among patient groups, it makes up the majority of elastin-targeting proteases in saliva. The total protease and NE-specific activity were measured in saliva samples based on the breakdown and release of fluorescent-conjugated elastin. Saliva samples were tested in duplicate from each patient group: HC $n=8$, BDq (quiet, no ulcer) $n=6$, BDa (active oral ulcer) $n=7$, RASq $n=5$, RASa $n=4$. **A.** The black bars represent each group's total protease activity based on the median fluorescence intensity. The grey

bars represent the level of NE-specific activity for the same saliva samples. Error bars are 5% of the group median. Porcine elastase was used as a positive control. Mann Whitney U test, * $p < 0.05$. Only significant differences are indicated. **B.** Percentage of NE activity relative to the total protease activity (group median values). Error bars indicate the interquartile region. The enzymatic ability of NE to digest elastin was similar in all groups regardless of ulceration.

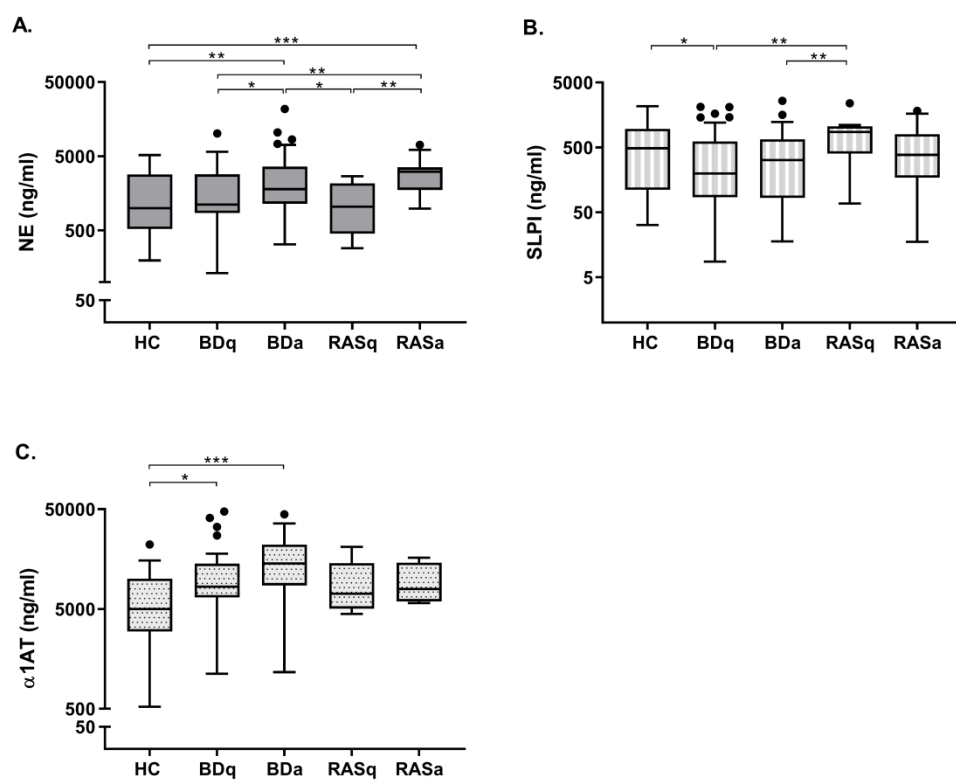
Figure 4. Mean SLPI mRNA Expression in Oral Buccal Swabs amongst Different Patient Groups. SLPI Cq normalised using PPIA as the reference gene and compared among HC (healthy control), RAS (recurrent aphthous stomatitis), BD (Behçet's disease). **q**, indicates quiet, no ulcers. **a**, refers to at least one active mouth ulcer regardless of other systemic symptoms. NU = swab taken from the buccal mucosa away from the oral ulcer. U = swab taken directly from the ulcer. Data analyzed using unpaired t-test with Welch's correction (2-tailed, 95% CI). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 5. Proposed Working Model of Behçet's Disease Oral Environment: Summary of the Interaction of the Epithelium, Neutrophil Elastase, SLPI and $\alpha 1$ AT. Individuals with BD have an increased presence of neutrophils in their vasculature which are recruited to the oral cavity due to hypersensitivity to ever-present microbes and proinflammatory cytokines (TNF- α , IL-8) produced by oral epithelial keratinocytes and immune cells. Fibroblasts and leukocytes further contribute IL-1 β which induces additional IL-8 production – a neutrophil chemoattractant. Neutrophils continue to migrate through the epithelium towards the site of accumulating inflammatory signals. Neutrophils phagocytose foreign entities or degranulate releasing NE into the extracellular environment. Activated neutrophils also release IL-8 thus reinforcing a cycle of inflammation. Neutrophils may apoptose shortly after controlled degranulation, but may become necrotic or undergo NETosis which releases NE non-discriminately. Salivary glands and epithelial cells work to produce SLPI to counteract the excessive NE, but SLPI is steadily used up. Although serum-derived $\alpha 1$ AT is carried to the oral cavity, there is no evidence that it can reduce NE activity. Neutrophils also have intracellular $\alpha 1$ AT and SLPI reserves, but the extent of their external inhibitory contribution is not known. Excessive, prolonged exposure to NE can directly damage epithelial cells and the integrity of the mucosal lining by breaking down ECM resulting in an ulcer. The injured tissue reinforces the inflammatory cycle and recruitment of neutrophils. Fluctuations of these inflammatory elements and reduced healing components may contribute to the recurrent ulcers seen in this disease. (Created using BioRender).

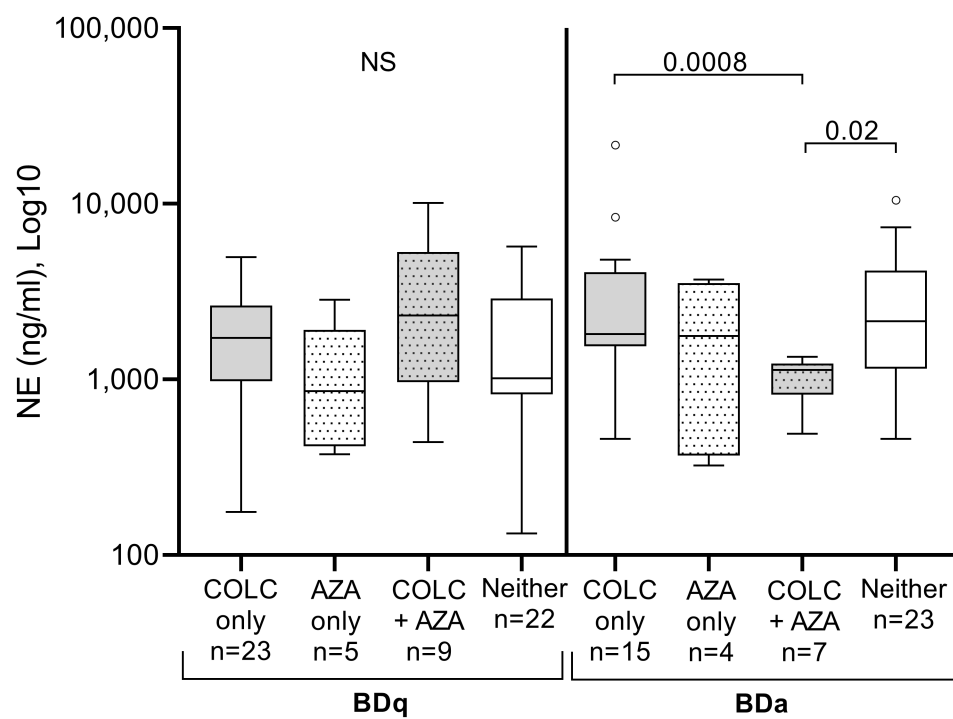
Table 1. Subject's demographics at the time of sample collection

NE ELISA	Subject Group	Total n	Age in years (Median, IQR)	Male (%)
	HC*	54	33 (29, 50)	33.3%
	BDq	59	38 (31, 49)	30.5%
	BDa	49	39 (29, 44)	36.7%
	RASq	10	42 (24, 45)	40%
	RASa	12	48 (40, 49)	58.3%
SLPI ELISA	Subject Group	Total n	Age in years (Median, IQR)	Male (%)
	HC*	49	32 (29, 48)	34.7%
	BDq	56	37 (31, 49)	33.9%
	BDa	52	39 (28, 47)	36.5%
	RASq	11	40 (27, 45)	36.4%
	RASa	15	47 (45, 49)	40.0%
α1AT ELISA	Subject Group	Total n	Age in years (Median, IQR)	Male (%)
	HC*	19	30 (27, 31)	36.8%
	BDq	29	43 (35, 49)	34.5%
	BDa	32	42 (29, 50)	31.3%
	RASq	5	35 (24, 47)	37.5%
	RASa	4	38 (27, 45)	25.0%

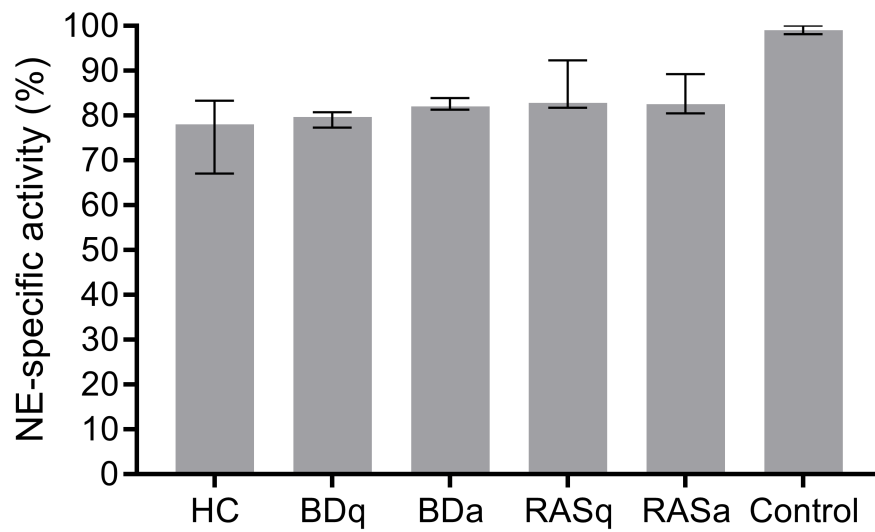
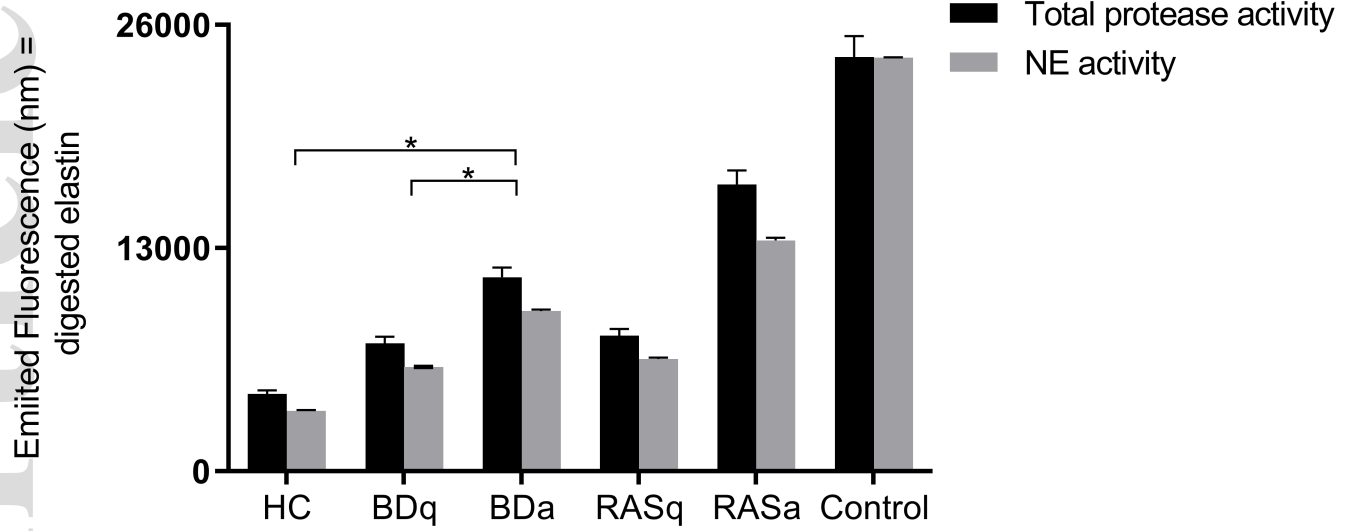
*HC NE ELISA had n=17 anonymous donors, SLPI n=10, α 1AT n=2
IQR, interquartile range



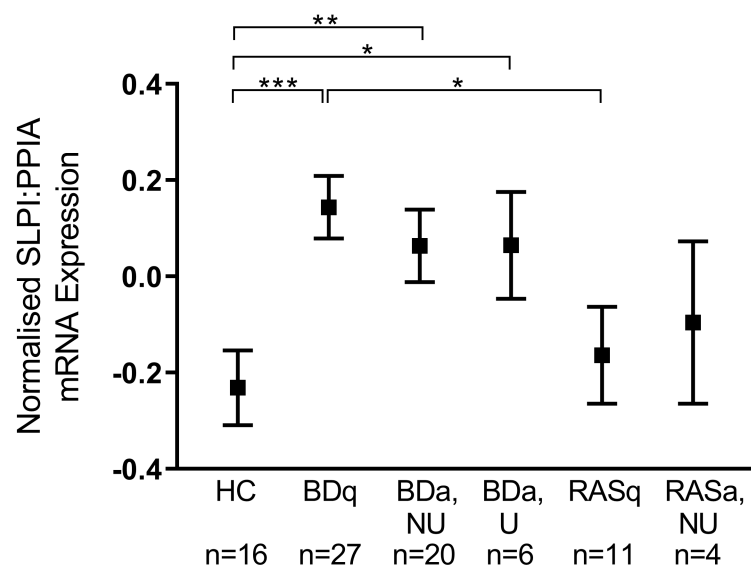
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